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SYNTHESIS AND CHARACTERIZATION OF SILVER (Ag) NANOPARTICLES AND ITS ANTIFUNGAL ACTIVITY AGAINST *Sclerotium rolfsii* IN CHILLI (*Capsicum annum* L.)

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ABSTRACT

Nanoparticles have renewed a great interest towards alternative methods of prevention and control of plant diseases, being widely used in the field of agriculture. It has the enormous potential in controlling the fungal pathogens. The size, shape and aggregation properties of the resultant nanoparticles were examined using scanning electron microscopy coupled with X-Ray diffraction, transmission electron spectroscopy and particle size analyzer. The measurement results indicated that the metal based silver nanoparticles (Ag NPs) were apparently smooth and with the size distribution was from 300 -350 nm. The chemically synthesized nanoformulations were tested for antifungal activities using poison food technique method. The nano formulation Ag was applied against the fungal pathogen *Sclerotium rolfsii* in chilli at various concentrations (0, 250, 500, 750, 1000, 1250, 1500 ppm). *In vitro* petri dish assay indicated that Ag NPs exhibited significantly higher antifungal activity against *Sclerotium rolfsii* at the concentration of 750 ppm by observing the growth of fungal hyphae and conidial germination. Further, efficient antifungal activity of the synthesized Ag NPs proves the application potential of nanoformulations against plant pathogens and its application on commercial scale needs to be exploited.

KEYWORDS: Antifungal Activity, *Sclerotium rolfsii*, Silver Nanoparticles, Chilli

INTRODUCTION

Chilli (*Capsicum annum* L.) is an important vegetable cum spice crop which is grown for both domestic and export market. It is a rich source of Vitamin C, A and B. India is the largest producer of chillies in the world (8.5 lakh tonnes) followed by China (4 lakh tonnes), Pakistan (3 lakh tonnes) and Mexico (3 lakh tonnes). Andhra Pradesh ranks first in India both in area and production with 2.04 lakh hectares producing 323 thousand tones. Chilli crop suffers with many fungal, bacterial and viral diseases resulting in enormous yield losses. Among the fungal diseases, in recent years dry root rot of chilli caused by *Sclerotium rolfsii* is of major concern and causing the high economic losses in chilli (Kalmesh and Gurjar 2001). It affects the yield severely whenever it occurs at any stage of the crop. Presently, greater emphasis should be placed on nanofungicides to control the soil borne pathogens and to avoid the development of resistant strains effectively. Hence, a holistic approach is formulated for the effective management and to examine the antifungal activity against *Sclerotium rolfsii* in chilli.

MATERIALS AND METHODS

Synthesis of Ag Nanoparticles

The Ag NPs were prepared by using chemical reduction method according to the description outlined with minor modification by Lee and Meisel (2005). Three different concentrations (1mM, 5mM, and 10mM) of $AgNO_3$ were prepared. Fifty milliliter of each concentration was taken in a separate beaker and boiled with hot plate. To this solution, 5ml of 1% trisodium citrate was added drop by drop from 10 ml measuring cylinder with vigorous mixing on the stirrer until pale yellow colour appeared. Among the three different concentrations, 5mM obtained higher NP's and smaller size of NP's. Then the beaker was removed and kept at ambient temperature where the chemical reaction occurred would have been

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4Ag0 + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2\uparrow$$

Characterization of Nanoparticles

Synthesized particles were characterized by using the following techniques described below.

Particle size analyzer (PSA)

Particle size, zeta potential and the distribution pattern of synthesized sample suspensions were determined using Horiba Scientific Nanopartica SZ-100 (Nanoparticle analyzer), Japan. Accurately, 0.5mg sample was dispersed in 20ml distilled water, sonicated for 15min and the suspension was analyzed under dynamic light scattering method using 90º or 173º at 25ºC.

X-ray Diffractometer (XRD)

The X-ray diffractograms was recorded on Powder XRD (Bruker D8 Advance Powder X-ray Diffractometer, Germany). This machine uses Cu-Kα radiation (0.154 nm) for measuring the crystalline nature of the material (Toraya, 1986). The diffractograms were recorded with 2θ value ranging between10-80 degrees at a scanning speed of 0.080 at a step time of 1s at room temperature (25ºC).

Scanning Electron Microscope (SEM)

SEM FEI QUANTA 250 was used to characterize the size and morphology of the nanoparticles. Sample of test nanoparticles (0.5 to 1.0 mg) was dusted on one side of the double sided adhesive carbon conducting tape and mounted on the 12 mm dia aluminum stub. Sample surface was observed at different magnifications and the images were recorded.

Transmission Electron Microscope (TEM)

TEM FEI TECHNAI SPRIT was used to analyze the sample. Dilute suspensions of nanoparticles (0.5 mg) in pure ethanol (15 ml) were prepared by ultrasonication. A drop of the suspension was placed on 300-mesh lacy carbon coated copper grid, dried and the images were recorded at different magnifications.

In vitro Assay of Antifungal Activity of Nano Fungicides

The antifungal activity of Ag nanoparticles were evaluated against *Sclerotium rolfsii* by using poisoned food technique *in vitro* using potato dextrose agar (PDA) medium amended with different concentrations (100, 250, 500, 750, 1000 and 1250ppm). The PDA medium without the amendment of nanoparticles served as a control. A nine millimeter disc of the actively growing pathogen of *Sclerotium rolfsii* from a 7 days old culture was placed at the centre in each of the nano particles amended medium as well as in the untreated check. The mycelial growth of the pathogen was measured after five

days of inoculation by incubating the Petri plates at 25 ± 5 ^{o}C. The per cent inhibition of the mycelial growth over control was calculated to express the antifungal activity.

Ultra Microscopic Changes on *Sclerotium rolfsii* Induced by Silver Nano Fungicides

Structural abnormality, hyphal lysis and inhibition of sclerotial formation induced by Ag nanoparticle were examined under SEM (Model FEI Quanta 250) at various resolutions (2500-6000X). The stub of SEM fixed with double-side adhesive carbon tape was gently pressed over the mycelia mat of Petri dishes having hampered growth, removed immediately and it was fixed in the appropriate location of the SEM for observing hyphal characters under low vacuum condition.

RESULTS AND DISCUSSIONS

Characterization of Silver Nanofungicides

Synthesized powders have been characterized for their morphology and particle size. From SEM results, Ag NPs with bundle of needle morphology ranging from 300 -350 nm (Fig. 1) respectively.

From particle size distribution study and TEM image analysis, it can be concluded that the particle size of Ag NPs ranged from 300 -350 nm with cylindrical and spherical morphology (Fig.**2**) respectively whereas the SEM measurements got enlarged 10 times. It is confirmed as nanometer in size with reference to earlier studies of Moghaddam *et al.* (2009); Sileikaite *et al*. (2006) and Arami *et al.* (2007).The diffraction pattern of TEM image shows a crystalline nature.

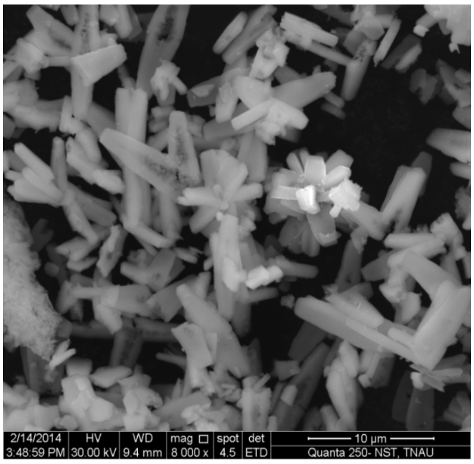

Figure 1: SEM Image of Silver Nanoparticles

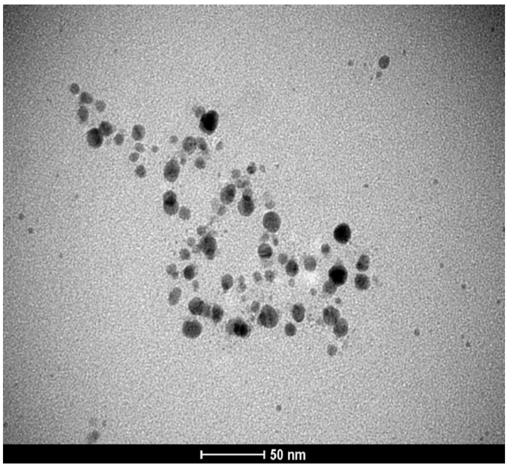

Figure 2: TEM Image of Silver Nanoparticles

XRD patterns of metal nanoparticles are also crystalline in nature which is confirmed by the observed intense peak (Fig. **3**) around 10 to 80° and the corresponding 2θ and d-spacing are in parthensis. The typical XRD pattern shown that the sample contained needle structure of Ag nanoparticles.

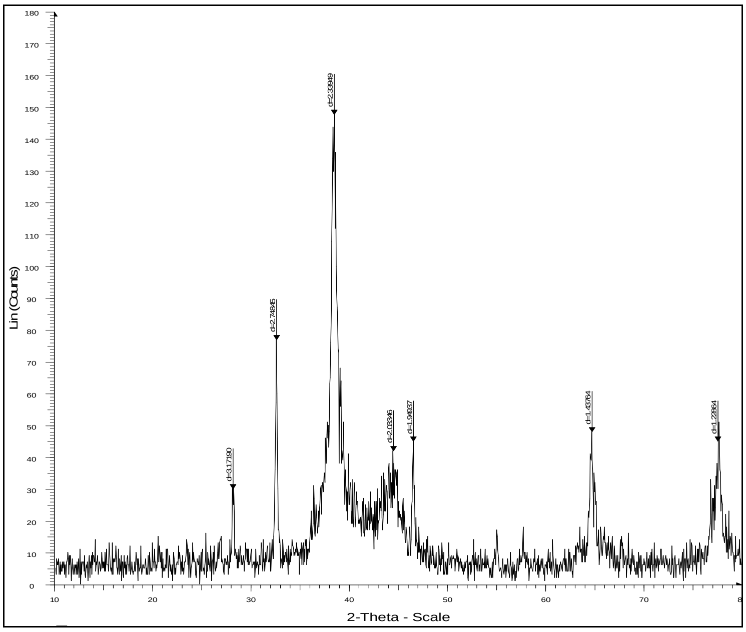

Figure 3: Powdered XRD Patterns of Synthesised Silver Nanoparticles (Ag)

Antifungal Activity of N ano Fungicides

Amending PDA medium with silver based nano particles at different concentrations indicated that Ag NPs was most effective in inhibiting the mycelial growth of *Sclerotium rolfsii* (Fig. 4). Inhibition of mycelial growth by Ag NPs was observed even at 0.1 per cent for the Ag Nps (Table 1). Besides inhibiting mycelial growth, Ag NPs induced mycelial malformations and inhibited sclerotial production at 100 and 250 ppm concentrations, which might reduce the ability of the pathogen to regenerate, multiply and cause disease. Exploitation of Ag NPs in future may pave for effective management of seed borne and soil borne pathogens like *Sclerotium* and thereby may reduce the pesticide load in the environment.

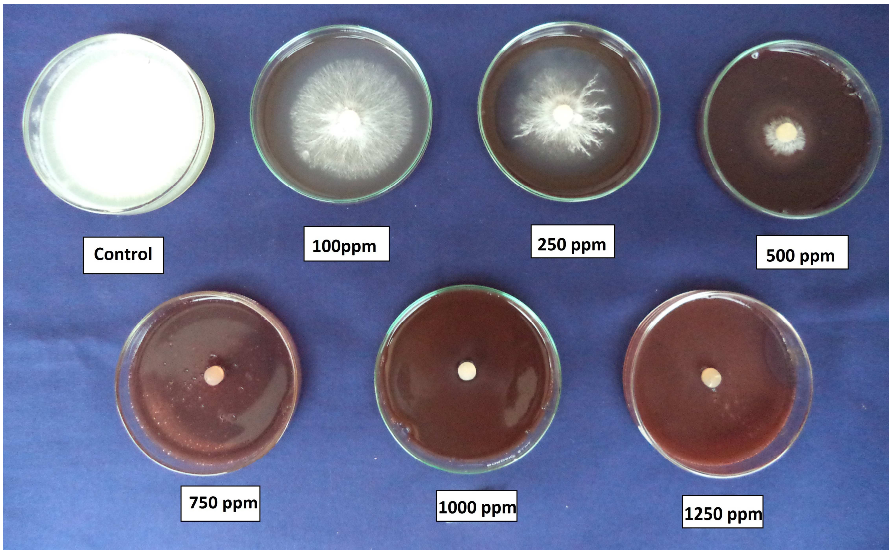

Figure 4: Antifungal Activity of AgNPs and Mycleial Growth of Sclerotium Rolfsii

Table 1: Mycelial Growth of *Sclerotium rolfsii* in PDA Medium Amended with Ag NPs

Nano particles (ppm Conc.)	Mycelial inhibition over control (%)
	Ag
100	25.3 d
250	58.5 c
500	91.7 b
750	100.0 a
1000	100.0 a
1250	100.0 a
Uninoculated control	3.3 e

Exploitation of nanoparticles as antifungal agents is relatively novice as being reported by recent workers (Kumar, 2011 and Sridhar, 2012). Nanoparticles interactions with fungal pathogen are dependent on the size and shape of the nanoparticles (Pal *et al*., 2007). Silver nanoparticles are an obvious choice due to their effective antimicrobial effects (Duncan, 2011). Among the tested dosages, the growth of *Sclerotium rolfsii* was greatly suppressed at 750 ppm concentration of Ag respectively; indicating antifungal activity of the synthesized nanoparticles as confirmed by the zone of inhibition growth. Increased surface areas of nanoparticles are reported to have the greatest antibacterial activity (Thiel *et al*., 2007). Thus, the results of the present study clearly revealed that the maximum level of inhibition zone was observed with the increasing concentration of nanoparticles.

Nanoparticles are highly antimicrobial and antioxidant to several species of bacteria, fungi and viruses. Probable mechanism as reported is expected that nanoparticles might interact with the outer membrane of fungi, and arrest the respiration and other metabolic pathways that leads to fatality of the fungi. Antimicrobial property of nanoparticles may be due to penetration of the cell wall and modulation of the cellular level signaling by dephosphorylating putative key peptide substrates, which are critical for cell viability and cell division (Shrivastava *et al.,* 2007). Nanoparticles are believed to inactivate microbial enzymes, facilitating production of reactive oxygen species that leads to microbial cell death (Allahverdiyev, 2011).

Morphological Modification of Mycelia

The mycelial fragment from nanoparticles treated and untreated plates were examined under SEM at various resolutions. Hyphal filament was smooth walled and equal in thickness throughout the length with blunt tips and found to bear sclerotial bodies as shown in Fig. 5(A) and (B). However, in nanoparticles treated plates, hyphae were found broken and sclerotial formation either lacking or abnormal, if formed. In addition, the cell surface of hyphae was observed to be crinkled as shown in Fig. 6(A), (B), (C) and (D).

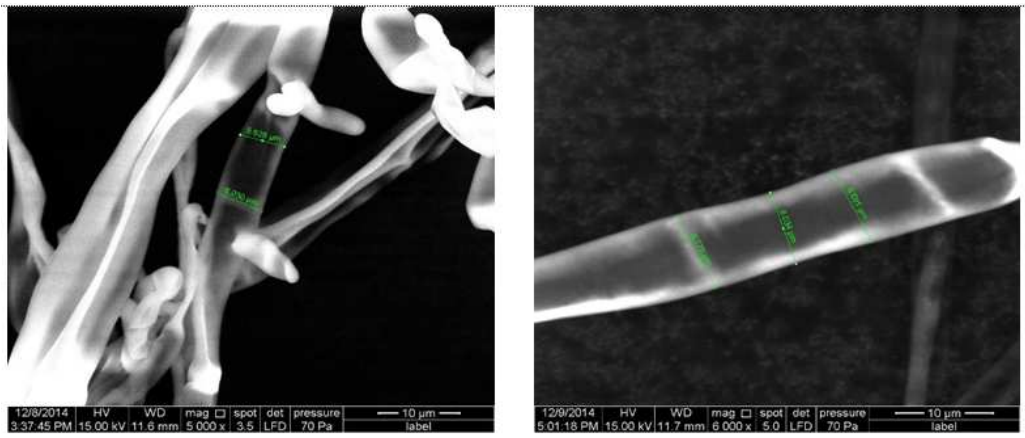

Figure 5: A & 5B. SEM Images of Sclerotium Rolfsii Showing Normal Mycleial Growth in

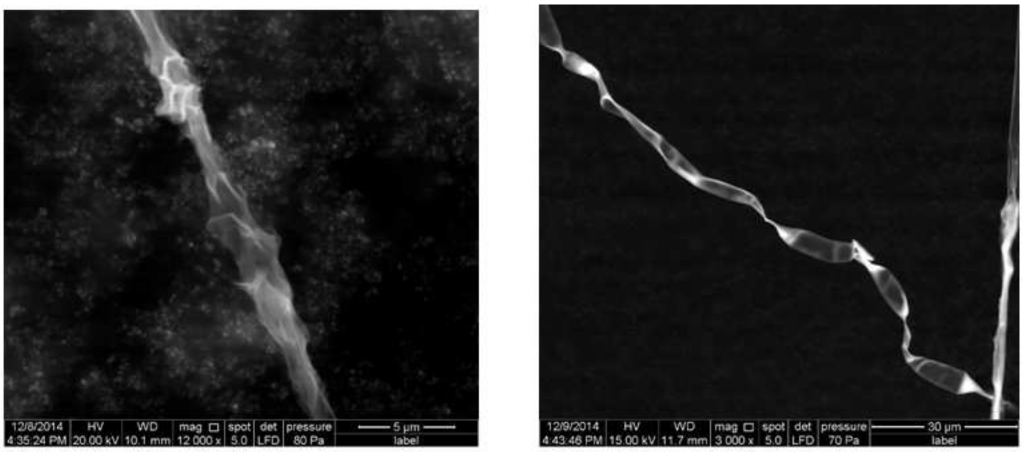

Figure 6: A and 6B. SEM Images of AgNP Treated Sclerotium Rolfsii Showing Abnormal Mycleial

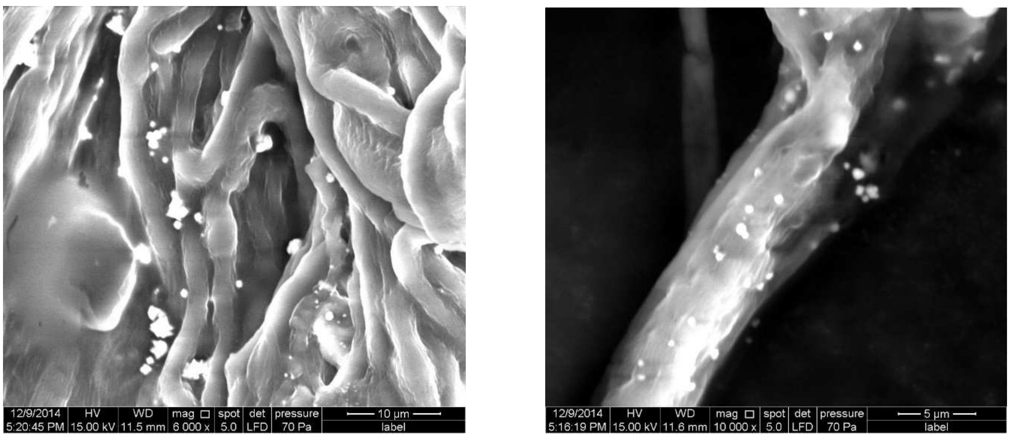

Figure 6C & 6D: Presence of Nanoparticles in the Mycelial Body

CONCLUSIONS

The present investigation is an offshoot of main purpose of exploring the possibilities of nano particles for increasing the seed qualities especially in chilli where the maintenance of seed viability is difficult task. Among the different concentrations, AgNPs at 750 ppm itself exerted better antifungal property when compared to control which may

due to inherent ability of Ag at cellular level besides the smallest size measuring 20-80 nm facilitating the easy reach to target locations Further AgO is also reported to have powerful antimicrobial activity. Hence, AgO may be considered in the crop production as one of the inputs for treating the seeds upon confirming the performance under field condition and subjecting to human safety tests.

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